Epidemiology and Genetic Analysis of Streptomycin-Resistant *Erwinia amylovora* from Michigan and Evaluation of Oxytetracycline for Control

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ABSTRACT

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Streptomycin-resistant strains of *Erwinia amylovora* were isolated from 14 of 63 Michigan apple orchards surveyed during 1991–1993. Orchards with resistant strains were located in three geographically distinct areas. The genes encoding streptomycin resistance, *strA* and *strB,* were usually located on DNA homologous to transposon Tn*5393* and to the self-transmissible plasmid pEa34. Ten percent of the resistant strains from one orchard contained DNA homologous to Tn*5393* on the chromosome or a resident plasmid, pEA29. Plasmids homologous to pEa34 but lacking Tn*5393* were rare in streptomycin-sensitive isolates, appearing in only three adjacent orchards 127 km from the nearest orchard with resistant strains containing pEa34. A streptomycin resistance mechanism unrelated to *strA* and *strB* was detected in all resistant isolates from two adjacent orchards and in 30% of the resistant isolates from a distant orchard, but not in resistant isolates from other orchards. Strains with the alternate mechanism were resistant to higher concentrations of streptomycin than were strains with *strA-strB.* In laboratory tests, streptomycin reduced populations of a streptomycin-sensitive strain of *E. amylovora* applied to the stigmata of apple flowers, but it did not affect populations of a streptomycin-resistant strain. Populations of both strains remained static on oxytetracycline-sprayed flowers and increased on water-sprayed flowers. Streptomycin was superior to oxytetracycline in reducing the incidence of blight on blossoms inoculated with a streptomycin-sensitive strain, but oxytetracycline was superior to streptomycin in reducing the incidence of blight on blossoms inoculated with a streptomycin-resistant strain.

Fire blight, caused by *Erwinia amylovora,* is the most devastating bacterial disease affecting apple (*Malus* × *domestica* Borkh.) and pear (*Pyrus communis* L.) trees. Streptomycin was first used to control phytopathogenic bacteria, including *E. amylovora,* in the late 1950s. After nearly two decades of intense use, strains of *E. amylovora* resistant to streptomycin were discovered in pear orchards in California, Oregon, and Washington in the early 1970s (6,17), and in apple orchards in Missouri in 1983 (26). Surveys conducted in New York and Michigan in the 1970s did not reveal streptomycin-resistant *E. amylovora* (1,30); however in 1990, streptomycin-resistant strains of *E. amylovora* were detected at one orchard in southwestern Michigan (4). Resistance at this orchard was mediated by aminoglycoside phosphotransferase enzymes encoded by *strA* and *strB,* genes carried by transposon Tn*5393*, which was inserted into the conjugative plasmid pEa34 (3–5). The genetic determinants of streptomycin resistance in strains from the western United States are apparently unrelated to *strA* and *strB* (4,18,24).

Streptomycin is the preferred bactericide for controlling fire blight, except in areas where streptomycin-resistant strains have emerged. Oxytetracycline has been used since the late 1970s to control fire blight in pear orchards in the western United States (19) and was recently approved by the Environmental Protection Agency (EPA) for use in Michigan in counties or townships where streptomycin resistance has been confirmed. Antibiotic sprays are recommended during bloom since, under favorable environmental conditions, *E. amylovora* multiplies rapidly on stigmata before infecting flowers (31). Although restricting multiplication of *E. amylovora* during bloom is pivotal in preventing blossom blight, the relative effectiveness of streptomycin and oxytetracycline for the control of blossom blight incited by streptomycin-resistant strains has not been demonstrated.

In this report we identify Michigan apple orchards containing streptomycin-resistant *E. amylovora* and identify the genetic determinants of streptomycin resistance in strains from these orchards. In addition, we compare the efficacy of streptomycin and oxytetracycline in restricting multiplication of *E. amylovora* on stigmata of apple blossoms and in controlling blossom blight.

MATERIALS AND METHODS

Bacterial strains. Streptomycin-resistant strain Ea88 of *E. amylovora,* isolated from a pear orchard in Washington, was provided by R. G. Roberts, Tree Fruit Research Laboratory, Wenatchee. Strain CA11, which was isolated from a crabapple orchard in Van Buren County, Michigan, is streptomycin-resistant due to *strA* and *strB* carried by transposon Tn*5393* in the self-transmissible plasmid pEa34 (4,5). All other strains of *E. amylovora* and streptomycin-resistant strain BC9 of *Erwinia herbicola* were isolated from apple orchards in Michigan.

Orchard survey. Sixty-three orchards in 12 counties throughout the major apple-producing regions of Michigan were sampled for streptomycin-resistant *E. amylovora* during June and July of 1991–1993. Only orchards which had a history of streptomycin use were sampled, but not all orchards were sampled every year. If resistant strains were detected in an orchard, then neighboring orchards were sampled. A blighted shoot, spur, or fruit comprised one sample, and approximately 25 samples were tested per orchard. Samples were transported to the laboratory in an insulated box and processed within 24 h.

Isolation and identification of *E. amylovora*. Bacterial ooze or small pieces of tissue from infected shoots, spurs, and fruits were streaked onto both King's medium B (KB) (11) supplemented with cycloheximide (KBc) at 50 μg/ml and KBc supplemented with streptomycin sulfate (KBsc) at 100 μg/ml. Plates were incubated at 28 C for 2–3 days. Nonfluorescent, cream-colored colonies typical of *E. amylovora* were transferred to either CG or CCT, the differential media of Crosse and Goodman (7) and Ishimaru

and Klos (9), respectively. Single colonies characteristic of *E. amylovora* on the differential media were transferred to KBc and KBsc to confirm their phenotype. Pathogenicity was determined by inoculating immature pear fruit. The identity of each isolate collected in 1992 and 1993 was confirmed with an *E. amylovora*-specific DNA probe (8) (see colony hybridizations, below).

Bacteria for population and blossom blight studies. A spontaneous mutant of streptomycin-sensitive strain G11 of *E. amylovora* resistant to nalidixic acid (G11nal^r) at 100 μg/ml was mated with streptomycin-resistant strain CA11 as described previously (4). Transconjugant strain G11nal^r(pEa34), which was genetically identical to G11nal^r except for the presence of pEa34, was used as the streptomycin-resistant strain in laboratory studies. Inoculum was prepared by suspending in sterile distilled water 48-h-old bacterial colonies from plates of KB amended with the appropriate antibiotics. The suspensions were adjusted to an optical density of 0.14 at 640 nm, which gave 2–4 × 10^8 cfu/ml.

Effect of antibiotics on populations of *E. amylovora* on stigmata and blossom blight incidence. In the population study, newly opened blossoms were cut at the peduncle from 2-yr-old potted apple trees (cultivar Gala, highly susceptible to fire blight) in the greenhouse. The peduncle of each flower was inserted through a hole in the cap of a sterile, disposable 5-ml plastic culture tube filled with sterile distilled water. Stigmata of 90 flowers were inoculated with 3 μl of a suspension of G11nal^r or G11nal^r(pEa34). Thirty flowers from each inoculation group were then sprayed until runoff with streptomycin (Agrimycin at 0.6 g/L) at 100 μg/ml, oxytetracycline (Mycoshield at 1.2 g/L) at 200 μg/ml, or water. These concentrations were the rates recommended on product labels for field application. Immediately after spraying and at 12-h intervals up to 60 h, the petals were removed from five replicate flowers of each treatment, the remaining flower parts were macerated in 1 ml of sterile distilled water, and serial dilutions were plated onto KBc supplemented with nalidixic acid (25 μg/ml) or nalidixic acid and streptomycin (100 mg/ml) as appropriate. Immediately after the initial sampling, the blossoms were enclosed in clear, plastic bags to maintain high humidities and were incubated at room temperature. This experiment was performed twice. Population doubling times were estimated from the slope of the regression of $\log_2$ (population size) vs. time after inoculation during the phase of most rapid population growth. To determine whether *E. amylovora* or other bacteria existed on uninoculated flowers from trees in the greenhouse, 10 fresh blossoms were macerated, and the homogenates were diluted and plated onto CGc or KBc.

In the blossom blight study, fruit spurs with newly opened blossoms were collected from apple trees (cultivar Jonathan, highly susceptible to fire blight) at the Botany and Plant Pathology Farm of Michigan State University at East Lansing. In the laboratory, the spurs were placed in 120-ml baby-food jars with water so that each jar contained 16–22 blossoms. The blossoms were sprayed until runoff with 0.6 g of iprodione plus 0.6 g of benomyl per liter of water to inhibit fungi. After the blossoms dried, they were inoculated with either G11nal^r or G11nal^r(pEa34), or mock-inoculated with water, by dipping a cotton swab into a suspension of the appropriate bacterial strain or water and touching the swab to the stigmata. Immediately after inoculation, five jars of blossoms from each of the three inoculation groups were sprayed until runoff with streptomycin (100 μg/ml), oxytetracycline (200 μg/ml), or water. The jars were randomized and incubated in a dew chamber at 28 C with 12 h of light per day provided by fluorescent bulbs. After 7 days, flowers were observed for symptoms of blossom blight. This experiment was performed twice.

Colony hybridizations. Putative isolates of *E. amylovora* collected in 1992 and 1993 were spotted onto nylon membranes (Amersham, Arlington Heights, IL) which had been placed on the surface of KBc 20 h earlier, and incubated at 28 C for 20–30 h. Colonies were lysed, the membranes were neutralized (10), and DNA was fixed to the membranes by baking at 80 C for 2 h. A 5-kb *Sal*I fragment (probe 5SUP) of pEA29, the 29-kb plasmid ubiquitous in and unique to *E. amylovora* (8,13), was purified by electrophoresis onto DEAE-cellulose paper (25) and labeled with digoxigenin-11-dUTP using the random primer method (Genius DNA Labeling and Detection Kit, Boehringer Mannheim Corp., Indianapolis, IN). Prehybridization, hybridization, and colorimetric or chemiluminescent detection of the probe were performed according to the manufacturer's instructions. A digoxigenin-labeled 0.5-kb *Bam*HI-*Ava*I internal fragment of Tn*5393* containing a portion of *strA* (5) (probe SMP3 [20]) was used to identify strains with *strA-strB*. The presence of pEa34, either with or without Tn*5393*, was detected with a digoxigenin-labeled 26-kb *Sma*I fragment of pEa34 (probe 26-Ea34). Plasmid and total genomic DNA from streptomycin-resistant isolates with DNA homologous to SMP3 but not to 26-Ea34 in colony hybridization was subjected to Southern analyses.

Isolation and Southern analysis of DNA. Indigenous plasmids of *E. amylovora* were isolated using the Magic Minipreps DNA Purification System (Promega, Madison, WI) and resolved on agarose gels (0.5% w/v) run at 5 V/cm in TAE electrophoresis buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Total genomic DNA was recovered by a miniprep procedure (33). Plasmid DNA for restriction analysis was purified by cesium chloride centrifugation (16). Southern analyses of intact plasmid DNA, restriction enzyme-digested plasmid DNA, and *Ava*I-digested total genomic DNA were performed using standard procedures (16). Probe SMP3 was used to identify the 2.7-kb internal *Ava*I fragment indicative of Tn*5393* in *Ava*I-digested plasmid and total genomic DNA (4,5). For multiple hybridizations, probes were stripped from membranes by gentle shaking in 0.5% sodium dodecyl sulfate (initially at 100 C, then allowed to cool to room temperature) and then rinsing briefly in distilled water. The insertion site of Tn*5393* into pEA29 was mapped by digesting pEA29::Tn*5393* with *Bgl*II, *Cla*I, *Hin*dIII, *Kpn*I, *Sal*I, and *Pst*I, probing Southern blots with SMP3, and comparing the results to published restriction maps of pEA29 (8,13).

Determination of minimum inhibitory concentration (MIC). MICs were determined by the agar dilution method (12). Bacteria were cultured overnight in LB broth (16) at 25 C with shaking and diluted 100-fold in sterile distilled water to ~10^7 cfu/ml. Three microliters of the diluted bacterial suspensions were spotted onto duplicate plates of KB amended with streptomycin sulfate at 0, 10, 50, 100, 500, 750, 1,000, or 2,000 μg/ml. The MIC was the lowest concentration of streptomycin sulfate that inhibited bacterial growth in the spots after 24 h incubation at 28 C.

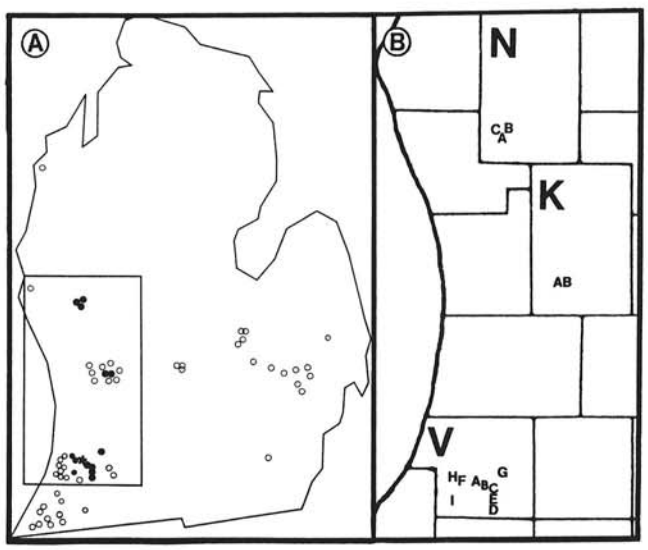

Fig. 1. Distribution of Michigan apple orchards surveyed for streptomycin-resistant *Erwinia amylovora,* 1991–1993. **A,** Orchards from which only sensitive strains were isolated (○), orchards harboring resistant strains (●), and orchard where resistance was first discovered in 1990 (☆) (4); boxed region is projected in B. **B,** Orchards harboring resistant strains in Kent (K), Newaygo (N), and Van Buren (V) counties. Orchard V-A is the original site of resistance.

RESULTS

Orchard survey. In 1991, streptomycin-resistant strains of *E. amylovora* were recovered from orchard V-A, the site where resistance was originally discovered in Michigan in 1990 (4), and from three other orchards within 2 km of V-A in Van Buren County (Fig. 1, Table 1). Also in 1991, resistance was detected at two adjacent orchards in Kent County, 94 km north-northeast of V-A. In 1992, streptomycin-resistant *E. amylovora* were found in six Van Buren County orchards, the same two in Kent County, and two Newaygo County orchards (132 km north of V-A). In 1993, streptomycin-resistant isolates were detected at all orchards listed in Table 1 except K-B and N-B. The frequency of streptomycin resistance (number of resistant samples/total number of samples × 100) ranged from 0 to 100% (Table 1). No resistant isolates of *E. amylovora* were detected at 49 of the 63 orchards surveyed (Fig. 1A).

Effect of antibiotics on populations of *E. amylovora* on stigmata and blossom blight incidence. Initial populations of streptomycin-sensitive G11nal^r and streptomycin-resistant G11nal^r(pEa34) were ~5.0×10^5 cfu per flower for all treatments (Fig. 2). After 12 h, populations of G11nal^r and G11nal^r(pEa34) on blossoms sprayed with water, and of G11nal^r(pEa34) on blossoms sprayed with streptomycin, increased to $3.2 \times 10^6 - 2.1 \times 10^7$ cfu per flower; populations peaked at ~6.0×10^7 cfu per flower by 36 h (Fig. 2). Populations of G11nal^r on blossoms sprayed with streptomycin decreased significantly by 48 h. Populations of G11nal^r and G11nal^r(pEa34) on blossoms sprayed with oxytetracycline were similar to each other and did not fluctuate significantly throughout the experiment. However, 12 h after inoculation, bacterial populations on oxytetracycline-treated blossoms were significantly lower than populations on water-treated controls. Similar results were obtained in a replicate experiment (data not shown). Population doubling times were similar on blossoms inoculated with either strain G11nal^r or G11nal^r(pEa34) following treatment with water (4.7 and 3.9 h). On streptomycin-sprayed blossoms, doubling times were 28.8 and 3.4 h for strains G11nal^r and G11nal^r(pEa34), respectively. Population doubling times on blossoms inoculated with strains G11nal^r or G11nal^r(pEa34) and then sprayed with oxytetracycline were 32.7 and 27.8 h, respectively. No *E. amylovora* were isolated from uninoculated blossoms from the greenhouse, and the number of unidentified bacteria ranged from 0 to 7 cfu/ml.

TABLE 1. Distribution and frequency of streptomycin-resistant (Sm^r) *Erwinia amylovora* in Michigan apple orchards relative to antibiotic spray history[a]

County	Orchard	Sm^r samples [Frequency[b] (Total no.) Spray history[c]] 1991	1992	1993
Kent	K-A[d]	>0 (≥6)[e]	13 (48)	4 (49)
			M, C	M, C
	K-B[d]	>0 (≥6)[e]	17 (18)	0 (1)[f]
		S:7	M:4	M:2
Newaygo	N-A	...[g]	100 (24)	48 (29)
		S:5, C:1	S:2, C:1	M:3, C:1
	N-B	...	21 (28)	0 (21)
		S:5, C:1	S:2, C:1	M:3, C:1
	N-C	...	...	43 (23)
				M
Van Buren	V-A	9 (11)	0 (28)	9 (47)
			M	M
	V-B	64 (11)	44 (27)	82 (62)
			M	S:4
	V-C	36 (11)	27 (37)	68 (22)
			M	S:3
	V-D	50 (4)	50 (8)	38 (16)
				S:3
	V-E	...	41 (22)	62 (13)
				M:3
	V-F	...	100 (7)	85 (39)
				S
	V-G[d]	...	18 (45)	6 (32)
		S:8	S:3, M:4	M:3
	V-H	...	...	84 (25)
				S
	V-I	...	...	92 (24)
				S

[a] Sixty-three orchards in 12 counties were sampled for streptomycin-resistant *E. amylovora*. Resistance was detected in three counties; data only for orchards where resistance was detected are presented. Streptomycin-resistant isolates from each orchard contained DNA homologous to probe SMP3 unless otherwise noted.

[b] Frequency (%) = streptomycin-resistant samples/total samples × 100.

[c] Data were based on personal communication with growers. S = streptomycin, M = oxytetracycline, C = copper, and blank = no definitive information on bactericide use available. Streptomycin was used in the past at all orchards. Number after a bactericide indicates number of applications; no number means number unknown.

[d] Probe SMP3 hybridized to DNA from 0% of streptomycin-resistant isolates from orchards K-A and K-B, and to 70% of resistant isolates from orchard V-G.

[e] Six streptomycin-resistant samples were isolated; total number of samples is unknown.

[f] Trees yielding streptomycin-resistant isolates were removed the previous year.

[g] ... = Orchard not sampled that year.

One-way analysis of variance of the blossom blight data indicated that the coefficients of variability for the two experiments were low and similar. Also, the F test between the error mean squares of the two runs showed that the error variances of the two runs were not significantly different ($P > 0.05$), indicating that the data from the two experiments could be pooled for further statistical analysis. Blossom blight incidence was >90% when stigmata were inoculated with streptomycin-sensitive G11nal^r or streptomycin-resistant G11nal^r(pEa34) and then sprayed with water (Fig. 3). The incidence of blight on blossoms inoculated with G11nal^r and then sprayed with streptomycin did not differ significantly from that of uninoculated blossoms. Blight incidence was comparable on streptomycin-sprayed blossoms and water-sprayed blossoms that had been inoculated with G11nal^r(pEa34). The incidence of blight on oxytetracycline-sprayed blossoms inoculated with G11nal^r or G11nal^r(pEa34) was similar but significantly higher than the incidence on uninoculated control blossoms, and significantly lower than the incidence on water-sprayed inoculated blossoms. None of 20 blighted blossoms from the uninoculated control group yielded nalidixic acid-resistant strains of *E. amylovora,* indicating that the blight resulted from epiphytic *E. amylovora* acquired in the field.

Colony hybridizations, plasmid profiles, and Southern analyses. Probe SMP3 (for *strA-strB*) hybridized to DNA from 0, 100, and 98.6%, and probe 26-Ea34 (for pEa34) hybridized to DNA from 0, 100, and 95.3% of the streptomycin-resistant strains of *E. amylovora* collected in 1992 and 1993 from orchards in Kent, Newaygo, and Van Buren counties, respectively. Ten percent of the resistant strains from orchard V-B contained DNA homologous to SMP3 but not to 26-Ea34, accounting for 3.3% of all resistant strains from Van Buren County. Thirty percent of the resistant strains from orchard V-G contained DNA that was not homologous to SMP3, accounting for 1.4% of all resistant strains from Van Buren County. None of the streptomycin-sensitive isolates contained DNA that hybridized to probe SMP3, while 11, 39, and 91% of the streptomycin-sensitive strains from three orchards in central Michigan, but none from the remaining 60 orchards, contained strains with DNA that hybridized to probe 26-Ea34.

Southern analysis of plasmid preparations of streptomycin-resistant and -sensitive strains of *E. amylovora* from various locations showed that each strain contained a plasmid homologous to probe 5SUP (for pEA29) (Fig. 4), while the plasmid of streptomycin-resistant strain BC9 of *E. herbicola* (Fig. 4, lane 12) did not hybridize to 5SUP. Streptomycin-resistant *E. amylovora* strains CA11, H6b, and M3a (lanes 1–3), and *E. herbicola* strain BC9 (lane 12) from Van Buren County, and streptomycin-resistant *E. amylovora* strain RN2b (lane 7) from Newaygo County, each had a 34-kb plasmid that hybridized to probes SMP3 and 26-Ea34. Streptomycin-resistant strains BCN12, BCN16,

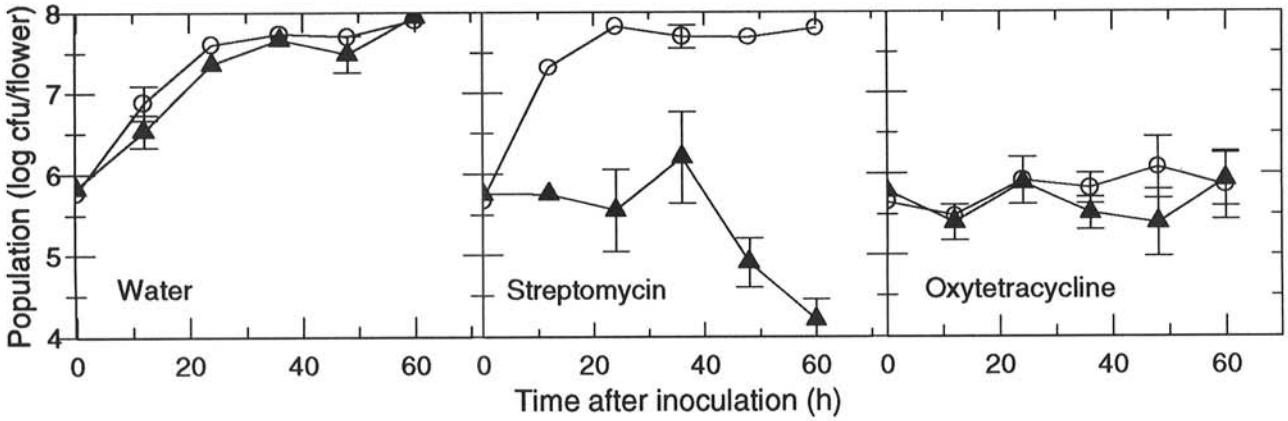

Fig. 2. Colonization of detached flowers of apple by streptomycin-sensitive *Erwinia amylovora* strain G11nal^r (▲) or streptomycin-resistant *E. amylovora* strain G11nal^r(pEa34) (○), after inoculation of stigmata and spraying with water, streptomycin (100 μg/ml), or oxytetracycline (200 μg/ml). Bars represent two standard errors of the mean.

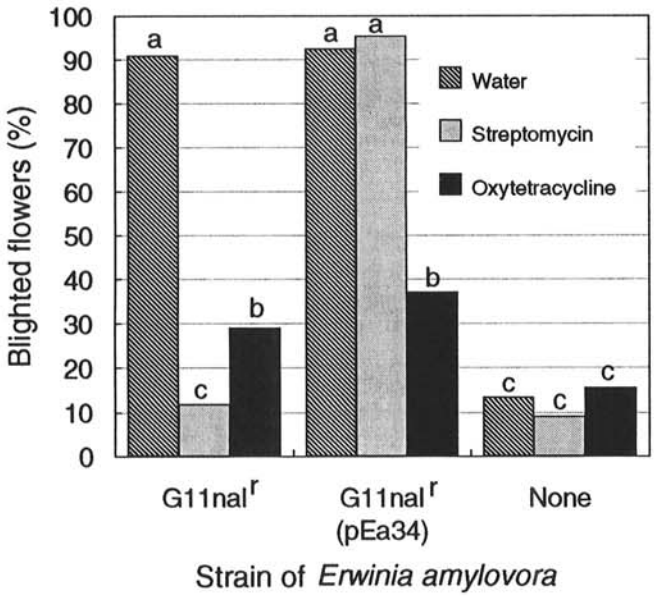

Fig. 3. Incidence of blossom blight in apple flowers 7 days after inoculation with strain G11nal^r (streptomycin sensitive) or G11nal^r(pEa34) (streptomycin resistant) of *Erwinia amylovora,* or mock-inoculation with water, and then spraying with water, streptomycin (100 μg/ml), or oxytetracycline (200 μg/ml). Letters above the bars denote significant differences ($P = 0.05$) between the means by least significant difference analysis, LSD = 8.9.

BCN20, and BCN87 (lanes 4–6 and 17), which showed DNA homology to probe SMP3 but not to probe 26-Ea34 in colony hybridizations, each lacked a 29- and 34-kb plasmid but contained a 36-kb plasmid (see below) homologous to both SMP3 and 5SUP. Streptomycin-resistant strains BCN74, BCN75, and BCN77 (lanes 14–16) each contained a 29-kb plasmid which hybridized only to probe 5SUP; although in colony hybridization, DNA from these strains showed homology to probe SMP3. Plasmid DNA of streptomycin-resistant strains BB8 and Ea88 (lanes 8 and 13) from Kent County and Washington, respectively, and of streptomycin-sensitive strains BCN27, PW23, and G11 (lanes 9–11) did not hybridize to either SMP3 or 26-Ea34.

Probe SMP3 hybridized to a 2.7-kb fragment in *Ava*I-digested plasmid and total genomic DNA from strains of *E. amylovora* or *E. herbicola* that contained a plasmid homologous to probe SMP3 (Table 2). Hybridization to a 2.7-kb fragment was detected in *Ava*I-digested total genomic but not in plasmid DNA from *E. amylovora* strains BCN74, BCN75, and BCN77, indicating that Tn*5393* was inserted into chromosomal DNA in these strains. Probe SMP3 did not hybridize to *Ava*I-digested DNA from streptomycin-sensitive strains. Probe SMP3 hybridized to a 2.7-kb *Ava*I fragment of the 36-kb plasmid in strains BCN12, BCN16, BCN20, and BCN87 (Table 2), demonstrating that pEA29 had acquired Tn*5393* to form the 36-kb plasmid pEA29::Tn*5393*. Restriction digests and Southern analysis of pEA29::Tn*5393* from strains BCN12, BCN16, BCN20, and BCN87 showed that the transposon was inserted into a region overlapped by 1.4-kb *Bgl*II and 1.6-kb *Hin*dIII fragments at approximately 27 kb from the previously designated *Bam*HI origin of pEA29 (8,13) (data not shown).

MICs. Streptomycin-resistant strains of *E. amylovora* lacking DNA homologous to probe SMP3 had MICs of streptomycin >2,000 μg/ml (Table 2). Bacteria containing plasmid DNA which hybridized to SMP3 had MICs of streptomycin at 750 μg/ml, whereas strains with total genomic but not plasmid DNA homologous to SMP3 had MICs at 500 μg/ml. Sensitive strains of *E. amylovora* were inhibited by streptomycin at 10 μg/ml.

DISCUSSION

Streptomycin-resistant strains of *E. amylovora* were isolated from 22% of 63 Michigan apple orchards surveyed during 1991–1993; the majority of orchards contained only streptomycin-sensitive strains (Fig. 1A). By contrast, Loper et al (15) reported streptomycin-resistant strains in 86% of the orchards throughout the major pear-growing regions in Washington. Streptomycin resistance probably emerged earlier and is more widespread in pear orchards in the West than in apple orchards in Michigan because of more intense use of streptomycin in the West (23,32).

Beginning in 1991, EPA regulations permitted growers in Van Buren County to use oxytetracycline for fire blight control. Growers used oxytetracycline in some orchards where resistance was confirmed but were reluctant to use it rather than streptomycin in nearby orchards where resistance had not been confirmed. Streptomycin-resistant *E. amylovora* continued to increase and spread in the county as the number of orchards with resistant strains increased from one (out of 20 orchards sampled) in 1990 (4) to nine in 1993. Moreover, the frequency of resistance was high at several Van Buren County orchards where streptomycin was applied in the presence of resistant strains (Table 1). In particular, at orchards V-B and V-C, the frequencies of resistance declined from 1991 to 1992 when oxytetracycline rather than streptomycin was used. However, populations of streptomycin-resistant *E. amylovora* quickly rebounded in 1993 when streptomycin use was resumed. Previously, Stall and Thayer (27) reported a rapid resurgence of streptomycin-resistant *Xanthomonas campestris* pv. *vesicatoria* following repeated application of the antibiotic in field studies on tomato in Florida. Thus, our data and those of Stall and Thayer underscore the importance of avoiding streptomycin as the primary bactericide at sites where streptomycin resistance has been confirmed. The proliferation of streptomycin-resistant *E. amylovora* in Van Buren County illustrates the impossibility of containing resistance in areas where

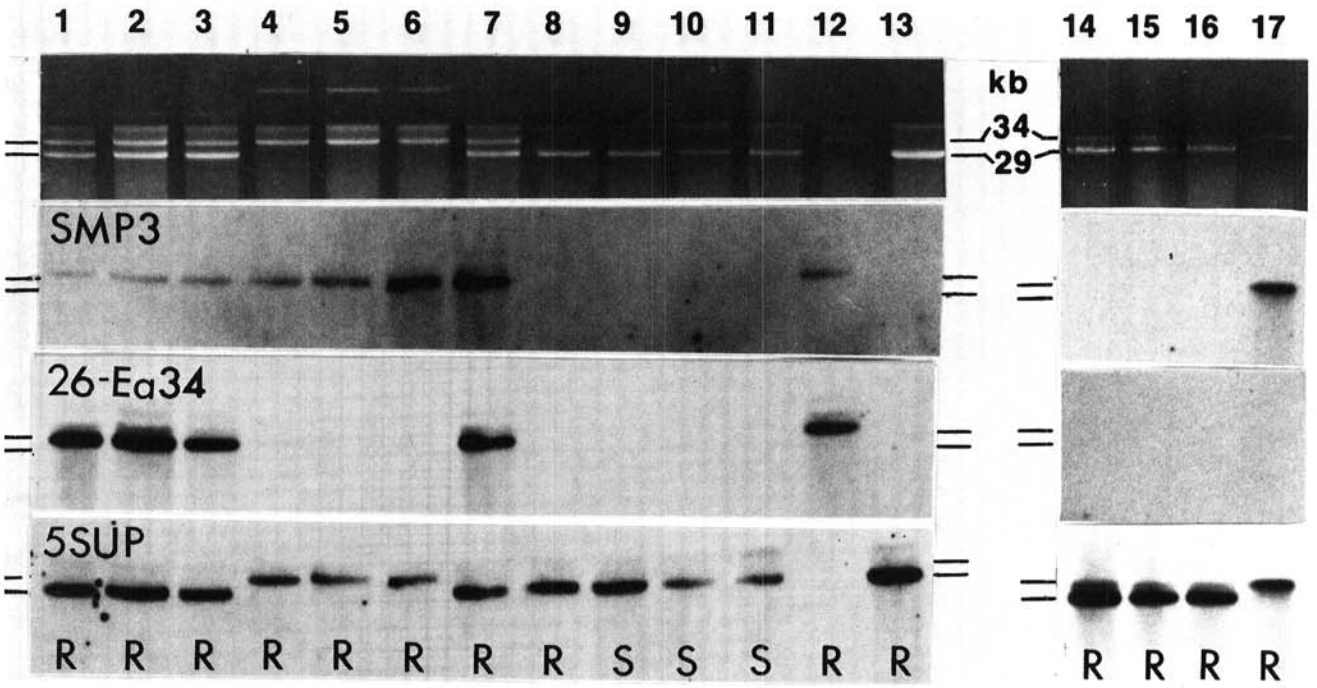

Fig. 4. Plasmid profiles and Southern analyses of streptomycin-resistant (R) and streptomycin-sensitive (S) strains of *Erwinia amylovora* (all lanes except lane 12) and a streptomycin-resistant strain of *Erwinia herbicola* (lane 12). Lane 1, CA11; lane 2, H6b; lane 3, M3a; lane 4, BCN12; lane 5, BCN16; lane 6, BCN20; lane 7, RN2b; lane 8, BB8; lane 9, BCN27; lane 10, PW23; lane 11, G11; lane 12, BC9; lane 13, Ea88; lane 14, BCN74; lane 15, BCN75; lane 16, BCN77; and lane 17, BCN87. The gel with lanes 14–17 included plasmids of strains CA11 and G11 as controls (not shown). The DNA was blotted and sequentially hybridized with probes SMP3 (for *strA-strB*), 26-Ea34 (for pEa34), and 5SUP (for pEA29). The positions of 29- and 34-kb plasmids are marked in all panels.

growers are unwilling to adopt alternate control strategies.

Streptomycin was superior to oxytetracycline in controlling colonization of stigmata by a streptomycin-sensitive strain of *E. amylovora,* but oxytetracycline was superior in controlling colonization by a streptomycin-resistant strain (Fig. 2). These data are consistent with the conclusion that the tetracycline derivatives are bacteriostatic, whereas streptomycin is bactericidal. The bactericidal activity of streptomycin vs. the bacteriostatic activity of oxytetracycline may explain why streptomycin was more effective than oxytetracycline in reducing blossom blight incited by a sensitive strain in our laboratory experiments (Fig. 3) and in field studies in New York (21). The failure of streptomycin and the success of oxytetracycline in inhibiting multiplication of and infection by G11nal^r(pEa34) illustrate the futility of attempting to combat streptomycin-resistant *E. amylovora* with streptomycin, and the value of oxytetracycline for controlling resistant strains. However, intense use of oxytetracycline could potentially result in the emergence of oxytetracycline-resistant strains of *E. amylovora.* While oxytetracycline-resistant strains were not detected among isolates of *E. amylovora* from 44 pear orchards in Washington (15), tetracycline-resistant bacteria are prevalent in the feces of pigs fed tetracycline-amended feed (14).

The clustering of orchards harboring streptomycin-resistant *E. amylovora* within each county, the finding that strains within a county shared a common resistance mechanism, and the geographic separation of the three regions with resistant strains indicate that resistance probably arose independently in each county. Certainly this was the case in Kent County, where a streptomycin resistance mechanism unrelated to *strA-strB* was detected. The alternate resistance mechanism detected in Michigan may be similar to the resistance mechanism in strains from the western United States, since strains from both locations had MICs >2,000 μg/ml (Table 2). The possibility of intercounty dispersal of *E. amylovora* with *strA-strB* on pEa34 mediated by humans, or introduction of the pathogen from a common source such as a nursery, cannot be eliminated. However, these scenarios are unlikely, since orchards in Newaygo County were managed independently of orchards in Van Buren County, and resistance would be ubiquitous in Michigan if nursery stock were contaminated with *E. amylovora* containing *strA-strB.*

Several lines of evidence suggest that plasmid-mediated streptomycin resistance originated from the conjugal transfer of pEa34 to *E. amylovora.* Some streptomycin-resistant strains of *E. herbicola,* a species ecologically associated with *E. amylovora,* contain pEa34 (Fig. 4) (5); and conjugal transfer of pEa34 from *E. herbicola* to *E. amylovora* and *Escherichia coli* has been demonstrated in laboratory mating studies (5). However, pEa34 is not ubiquitous among bacteria associated with apple trees, since DNA from 142 epiphytic gram-negative bacteria with *strA-strB* did not hybridize to probe 26-Ea34 (5). It is likely that pEa34, and not just Tn*5393,* was transferred to *E. amylovora,* because streptomycin-sensitive strains containing a plasmid homologous to probe 26-Ea34 are rare; they were found in only three adjacent orchards in central Michigan and have not been reported in populations of *E. amylovora* originating outside Michigan (28). Thus, we believe pEa34 moved into *E. amylovora* relatively recently, and our data pertain to streptomycin resistance during its early stages in Michigan.

Whereas pEa34 is rare in gram-negative bacteria from apple orchards, Tn*5393* is widespread among streptomycin-resistant gram-negative bacteria in Michigan (5). In New York, DNA from streptomycin-resistant *Pseudomonas syringae* pv. *papulans,* strains of *Pseudomonas* spp., and unidentified yellow bacteria associated with fire blight infections hybridized to probe SMP3, but no resistant *E. amylovora* were detected (2,20). Minsavage et al (18) identified plasmid-borne streptomycin-resistance genes in *X. c. vesicatoria* that were related to those of *P. s. papulans* strain Psp36 and speculated that a transposon was involved in the insertion of these genes on the chromosome of *X. c. vesicatoria* strain 87-77 from Ohio. Sundin and Bender (29) proposed that transposition might account for the presence of *strA-strB* homologs on plasmids of various sizes in *Pseudomonas syringae* pv. *syringae* from Oklahoma. Thus, *strA-strB,* and possibly Tn*5393,* are widely dispersed among phytobacteria in the United States.

Strains containing pEA29::Tn*5393,* or containing Tn*5393*

TABLE 2. Minimum inhibitory concentrations (MICs)[a] of streptomycin and detection of Tn*5393*[b]

Species Strain	MIC (μg/ml)	Orchard of origin[c]	Location of Tn*5393*[b]: Plasmid DNA	Location of Tn*5393*[b]: Total genomic DNA
Erwinia amylovora				
CAll	750	V-A	+	+
H6b	750	V-F	+	+
M3a	750	V-D	+	+
RN2b	750	N-B	+	+
BCN12	750	V-B	+	+
BCN16	750	V-B	+	+
BCN20	750	V-B	+	+
BCN87	750	V-B	+	+
BCN74	500	V-B	−	+
BCN75	500	V-B	−	+
BCN77	500	V-B	−	+
BB8	>2,000	K-B	−	−
Ea88	>2,000	...[d]	−	−
BCN27	10	V-B	−	−
PW23	10	...[d]	−	−
G11	10	...[d]	−	−
Erwinia herbicola				
BC9	750	V-B	+	+

[a] MICs were determined by spotting 3 μl of an $\sim 10^7$ cfu/ml suspension of each strain onto King's medium B amended with streptomycin sulfate at 0–2,000 μg/ml.

[b] Hybridization of probe SMP3 to a 2.7-kb internal *Ava*I restriction fragment was indicative of Tn*5393* (4,5).

[c] All orchards contained streptomycin-resistant *E. amylovora* and were located in Kent, Newaygo, or Van Buren counties (Fig. 1) unless otherwise noted.

[d] Ea88 was from Washington State; PW23 was from an orchard in southeastern Michigan lacking streptomycin-resistant *E. amylovora*; G11 was from an orchard in Van Buren County lacking streptomycin-resistant *E. amylovora*.

inserted on the chromosome, lacked pEa34, suggesting that after transposition of Tn*5393*, the strain lost pEa34 in subsequent generations. Alternatively, a plasmid other than pEa34 might have been transferred to *E. amylovora* and delivered Tn*5393*, but was unable to survive in its new host. Although we did not demonstrate transposition of Tn*5393* from pEa34 to pEA29 or to the chromosome, the occurrence of Tn*5393* on genetic elements common to all *E. amylovora* is evidence that the streptomycin-resistance transposon can reside on genetic elements that are stable in the species and is not associated exclusively with a conjugative plasmid.

Strains of *E. amylovora* carrying Tn*5393* on the chromosome probably have fewer copies of *strA-strB* than strains with pEa34 or pEA29::Tn*5393*. This could account for lower MICs among strains with Tn*5393* inserted on the chromosome compared to strains with plasmid-borne Tn*5393* (Table 2).

Tn*5393* was inserted at the same site on pEA29 in all four strains containing pEA29::Tn*5393*, possibly because several clones arose following one insertion event. Previous laboratory studies showed that Tn*5393*, a Tn*3*-type transposon, inserted at different sites on plasmids in *E. coli* (5). Other than a preference for AT-rich regions, Tn*3*-type transposons show little target-site specificity (22); thus, Tn*5393* probably inserts into other sites on pEA29. Plasmid pEA29 is not required for pathogenicity, but its genes are believed to modulate the development of fire blight symptoms (8,13). Strains harboring only pEA29::Tn*5393* were similar to strains with both pEA29 and pEa34 in MIC (Table 2), pathogenicity, and growth rate in liquid culture (data not shown), indicating that the insertion had not disrupted genes involved in these functions.

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